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Adhesion of *Pseudomonas putida* KT2442 Is Mediated by Surface Polymers at the Nano- and Microscale

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ABSTRACT

Pseudomonas putida KT2442 is a bacterium with potential for use in bioremediation of chlorinated hydrocarbons in soils, and its behavior in the subsurface is believed to be controlled by surface polymers. The role of surface macromolecules on the retention and adhesion behavior of this bacterium was examined by bacterial treatment with cellulase, an enzyme that breaks down the $\beta(1 \rightarrow 4)$ linkages of cellulose and similar macromolecules. Enzymatic treatment involved centrifugation to separate bacteria from suspending media. Two types of control systems were evaluated, namely, cells separated from growth media via filtration and centrifugation. Bacterial retention was quantified by the collision efficiency, α, the fraction of collisions that result in attachment. In batch retention studies (to glass), both controls had the same α values (1.19 ± 0.25, 1.20 ± 0.23, for filtered and centrifuged cells, respectively). In column transport/retention assays (to quartz), αs for the control groups were not statistically different from one another (0.34 ± 0.06, 0.45 ± 0.07, for filtered and centrifuged cells, respectively; data fails Mann-Whitney Rank Sum test). Treatment with cellulase decreased cell retention in both systems. The α values were decreased by 40% for cellulase-treated cells in batch tests, to 0.69 ± 0.13, and in column tests, cells treated with cellulase had α values below those from either control group (0.21 ± 0.05). Retention was correlated with nanoscopic adhesion forces measured with an atomic force microscope (AFM), as treatment with cellulase decreased adhesion forces from 1.05 ± 0.07 to 0.51 ± 0.03 nN. These results suggest that surface modification of *P. putida* KT2442 with cellulase alters adhesion/retention properties at the batch, column, and nanoscale, due to removal of polysaccharide material.

Key words: bacterial adhesion; polysaccharide; biopolymer; biofilm; bioadhesion; bacterial interaction forces; bacterial transport

INTRODUCTION

KNOWLEDGE OF THE INITIAL ADHESION events between a bacterium and a substrate are critical to understanding biofilm formation (Marshall *et al*., 1971). While electrostatic repulsive forces between a bacterium and substrate may make it difficult for contact between the two to occur, biomacromolecules on the bacterial surface may easily protrude through the repulsive layer (Simoni *et al*., 1998). Bacterial polymers can affect adhesion

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affinities of bacterial cells in numerous ways, depending on the biopolymers' chemical compositions (Rijnaarts *et al*., 1995; Jucker *et al*., 1997; Takashima and Morisaki, 1997), size (Ong *et al*., 1999; Burks *et al*., 2003), conformation (Mcwhirter *et al*., 2002; Abu-Lail and Camesano, 2003b), charge (Rijnaarts *et al*., 1995), and spatial density and distribution (Abu-Lail and Camesano, 2002; Camesano and Abu-Lail, 2002).

After the initial adhesion step, biofilm formation requires other biological events to occur, such as the release of extracellular DNA (Whitchurch *et al*., 2002), the expression of specific surface proteins such as curli (Vidal *et al*., 1998; Prigent-Combaret *et al*., 2000), or cell-to-cell signaling (Stoodley *et al*., 2002). However, strategies for minimizing biofilm formation often focus on prevention of the initial bacterial adhesion step (Bajeva *et al*., 2004).

Bacterial adhesion affects a number of processes relevant in environmental engineering, including *in situ* bioremediation, since only bacteria that are not retained by the soil surfaces can become dispersed throughout the contaminant plume. Bacterial retention onto soil grains has been found to impede successful bioaugmentation (Deflaun *et al*., 1997; Dong *et al*., 2002a). Other environmental processes affected by bacterial adhesion and biofilm formation include microbial extraction of trace metals from minerals (Bolton and Gorby, 1995; Brantley *et al*., 2001), microbial dissolution of minerals (Maurice *et al*., 2001), and the potential contamination of drinking water supplies via mobile microbial pathogens (Jiang *et al*., 2002). Most prokaryotic cells in natural systems are associated with surfaces, with the number of prokaryotes associated with aquifer sediments estimated to be several orders of magnitude greater than the number of unattached cells found in groundwater (Whitman *et al*., 1998).

Although this has not been verified experimentally, it has been suggested through mean-field calculations that even a single polymer on a bacterial surface can govern whether attachment occurs, by causing steric repulsion great enough to overcome van der Waals attraction and prevent bacterial adhesion to a surface (Cloud and Rajagopalan, 2003). Yet, experimental observations of bacterial attachment are often higher than would be expected based on measurements of significant steric repulsive forces in atomic force microscopy (AFM) experiments (Abu-Lail and Camesano, 2003c), which may suggest that some highly adhesive polymers can protrude through the steric barrier. Since the roles of polymer-induced steric interactions on bacterial adhesion are still not clear, there exists a need to better characterize the properties of bacterial polymers at the level of individual molecules. Recent progress in AFM and single-molecule force spectroscopy (SMFS) allow for the probing of biomolecules on bacterial surfaces at nanoscale resolution (Lower *et al*., 2000; Van der AA *et al*., 2001; Abu-Lail and Camesano, 2003a).

The objective of this study was to demonstrate the link between surface polysaccharides and the adhesion/retention properties of *Pseudomonas putida* KT2442, interacting with several model surfaces at different scales. Previous work with this strain has demonstrated long, rigid surface polymers, which were speculated to be cellulose or similar to cellulose in their structure (Camesano and Abu-Lail, 2002). Enzymatic digestion was used to remove cellulose and related molecules from the bacterial surfaces, and SMFS was used to detect individual polysaccharide molecules. In the process of treating the bacterial cells with an enzyme, several steps of separating the bacterial cells from the surrounding media were needed. Therefore, a complementary goal of this research was to distinguish between effects on cell retention due to the surface polymers, and effects caused as artifacts of the separation technique employed. The two "control" cases we considered were very gentle centrifugation and separation by capturing bacterial cells onto a syringe filter and manually backwashing the filter into ultrapure water. Nanoscale adhesion forces measured with the AFM were correlated with bacterial retention at the microscale in batch retention experiments and in packed-column assays with quartz sand as the medium.

MATERIALS AND METHODS

Culture of bacteria

This study addressed the retention and adhesion properties of *Pseudomonas putida* KT2442, a flagellated, Gram-negative, rod-shaped bacterial strain, of interest in environmental engineering for its ability to significantly degrade chlorinated hydrocarbons in soil systems (Nüßlein *et al*., 1992). KT2442 is the rifampicin-resistant derivative of strain KT2440, and the two are used extensively as hosts for the cloning and manipulation of genes from soil bacteria (Timmis, 2002). *P. putida* KT2442 was provided by D.F. Dwyer (Department of Earth, Ecological, and Environmental Sciences, The University of Toledo, Toledo, OH). A frozen preculture of a concentrated cell solution (1 mL) was thawed and added to 5 mL of tryptic soy broth solution (0.03 g/l). This culture was grown at 25°C and 50 rpm on a test-tube rotator for approximately 24 h, at which time, a 1-mL portion was transferred to a 100-mL solution of M9 buffer containing mineral salts, with 5 mM benzoate as the substrate and 50 μg/L rifampicin (Nüßlein *et al*., 1992). The culture was incubated at 25°C in a horizontal shaker bath until late exponential growth stage, which occurred after

approximately 16–18 h, and corresponded with an optical density of 0.5–0.6 at 600 nm.

Enzyme treatments

In some experiments, KT2442 cells were treated with cellulase to test how removal of some surface molecules would affect the adhesive properties of the bacteria. Cellulase from *Trichoderma reesei* ATCC 26921 (Sigma, St. Louis, MO) was employed. Cells grown to exponential phase (as described above) were centrifuged at 1125 rpm (190 g) for 10 min, then washed and resuspended in an equal amount of 20 mM MES buffer solution (pH = 6.44). The cell sample was diluted to 2×10^8 cells/mL in MES buffer, and the enzyme cellulase was added (4 mg/mL for a 60-mL sample of bacterial solution). The 60-mL sample was agitated at 50 rpm for 1 h on a horizontal shaker table (25°C). These treated cells were again washed with an equal volume of MES buffer solution and resuspended in an equal amount of ultrapure water (milli-Q water; Millipore Corp., Bedford, MA) via two subsequent centrifugation steps (190 g, 10 minutes each). Therefore, enzyme-treated cells were subjected to a total of three centrifugation steps.

Biochemical assays

To determine the mechanism of cellulose action on the cells, we performed a biochemical assay using the anthrone reagent, a colorimetric test to determine total carbohydrate content of a sample (Calza *et al*., 1985). Fifty milligrams of anthrone reagent were dissolved in 23.75 mL of concentrated sulfuric acid and 1.25 mL of distilled water. A 0.5-mL sample of bacterial polymer-containing solution was mixed with 1.0 mL of the anthrone solution and heated for 10 min at 100°C. Upon cooling, the absorbance was measured at 630 nm, using glucose for calibration.

Microscopic studies

Filtration and centrifugation controls. As controls, KT2442 cells were separated by either centrifugation or backfiltration onto a syringe filter. Centrifugation was identical to the procedures described for enzymatic treatment, except that no enzyme was added. As an alternative to centrifugation, the cells were captured (manually) onto a 0.45-μm membrane (Tuffryn®) of a 25 mm syringe filter (Acrodisc®). The filter was washed with ultrapure water, reversed, and the bacterial cells were backwashed to resuspend them in an equal amount of ultrapure water.

Batch retention of bacterial cells on glass. In the batch retention assays, treated, filtered, and centrifuged KT2442 cells were allowed to adhere to glass slides to determine the number of cells retained on glass and to quantify the collision efficiencies. Glass microscope slides (VWR Scientific, West Chester, PA, plain micro slides, 3" × 1" × 1 mm) were cut into thirds and cleaned with a 3:1 hydrochloric acid and nitric acid solution, then a 4:1 sulfuric acid and hydrogen peroxide solution, each for 25 min. One of these slides was immersed in a reactor containing 20 mL of bacterial solution (1×10^7 cells/mL) for a given reaction time, at ambient temperature.

In preliminary experiments, we needed to determine that diffusion was the mechanism of cellular transport from the bulk media to the glass. Therefore, batch assays on the centrifuged-only KT2442 cells were performed using reaction times of 30, 60, 120, and 240 min. If the surface coverage increases linearly as $t^{1/2}$ increases, then it is appropriate to model the process as molecular diffusion. We verified that this was the case using KT2442 from one of the control groups (centrifuged only; Fig. 1). In subsequent experiments, we used only a single time point of 120 min for all the reactors. This simplification was made because this reaction time resulted in a moderate number of bacterial cells per field, which could be easily and reproducibly counted under the microscope. In addition, the 120-min reaction time was modeled after similar experiments with *Pseudomonas aeruginosa* PAO1 and *Desulfovibrio desulfuricans* (ATCC 29577; Medilanski *et al*., 2003).

After the reaction time, the slides were carefully removed from the beakers with tweezers, rinsed with ultrapure water (2 mL), stained with an acridine orange solution (200 μL of 1 g/L acridine orange, 2% formaldehyde) for 10 min, and mounted onto a glass microscope slide. Slides were examined with an E400 Nikon Eclipse microscope using oil immersion (100×), to obtain the average number of bacteria/field. At least 10 fields/slide were counted, with the number of bacteria/field ranging from ~15 to 45. The average and standard error of the mean cell counts were calculated for each slide, as well as the number of cells retained/cm^2.

These values were then used to determine the collision efficiency (α) for each experiment. The final α values reported for each condition represent the average α obtained for the triplicate reactors. The collision efficiency is the fraction of bacteria that stick to the surface compared to the number of bacteria that come in contact with the surface. When diffusion is the only transport mechanism by which the bacteria can come into contact with the surface, α is calculated from (Smet *et al*., 1999).

$$N_t = 2\alpha C_b \sqrt{Dt/\pi} \quad (1)$$

where N_t is the retention count for the glass slide in

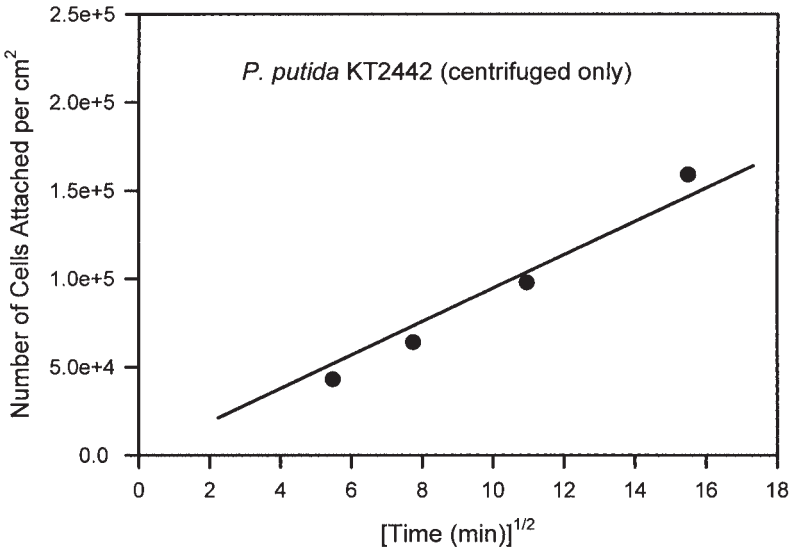

Figure 1. Results of batch adhesion assays for *P. putida* KT2442 cells that had been only centrifuged (not treated with an enzyme) in ultrapure water. The surface area covered by the cells increased linearly with $t^{1/2}$, implying that diffusion is the transport mechanism for the bacterial cells. The solid line represents the linear regression of the data, and must pass through the origin, according to Equation 1.

cells/m^2, C_b the concentration at the start of reaction in number of cells/m^3 ($\sim 1 \times 10^{13}$ cells/m^3 in our experiments), t the time of reaction in seconds, and D the diffusivity in m^2/s, given by Stoke's Law,

$$D = (k_B\ T)/(6\ \pi\ \mu\ a_p) \tag{2}$$

where k_B is the Boltzmann constant (1.38×10^{-23} J/K), T is absolute temperature, μ the viscosity (8.94×10^{-4} N $\cdot$ s/m^2), and a_p the radius of the bacterium (4.0×10^{-7} m). The bacterial equivalent radius was determined by performing image analysis on at least 50 bacterial cells. Images were of acridine orange-stained bacterial cells (Hobbie *et al*., 1977), captured onto membrane filters.

Column experiments. The transport of treated, filtered, and centrifuged KT2442 cells through porous media was quantified during steady-state flow through triplicate packed columns [Sigma white quartz sand; collector diameter (d_c) = 3.27×10^{-4}m]. Columns were fabricated from acrylic (10 cm high, diameter = 1.2 cm). All tubing was Tygon (R-3603; 5 mm diameter), and a peristaltic pump supplied flow to the column in the upflow direction (Vera Variastaltic Pump Plus by Manostat, New York, NY). Each column was tightly packed with dry white quartz sand using the tap and fill method. Ten pore volumes (PV) of ultrapure water were passed thorough the column at 0.022 mL/s followed by addition of the bacterial solution (10 PV, 1×10^7 cells/mL) and a 5 PV rinse with ultrapure water. The column effluent was collected during the bacterial injection and rinse phases. Two-milliliter samples from the effluent were diluted (1:10 or 1:20 depending on the conditions), stained with acridine orange (200 μL), and filtered onto 0.2 μm black filters (Millipore Isopore Membrane Filters GTBP) using vacuum filtration. The filters were mounted onto glass microscope slides and imaged via fluorescence microscopy (E400 Nikon Eclipse) using an oil immersion lens (100×). The average number of bacteria per field was quantified by examining at least 10 fields per filter (Hobbie *et al*., 1977). Effluent concentrations (C) were calculated and normalized to the initial concentration of bacterial injected into the column ($C_o \cong 1 \times 10^7$ cells/mL). The total fractional retention of bacteria to the sand was taken as $F_R = 1 - C/C_o$. We calculated collision efficiencies for the column studies using the Yao *et al*. (1971) model,

$$\alpha = -\frac{2}{3}\ \frac{\ln(1 - F_R)}{(1 - \theta)\eta L} \tag{3}$$

where θ is the porosity of the medium, L is the length of the column, and η is the collector efficiency.

The collector efficiency was calculated by two methods, using one expression developed by Rajagopalan and Tien (1976) (RT model) and another developed by Tufenkji and Elimelech (2004) (TE model). The RT model is a semiempirical solution to the trajectory analysis describing flow of a spherical particle towards a spherical collector, and is based on a numerical solution

of the trajectory equation for non-Brownian particles. The recently proposed TE model is based on a numerical solution of the convective–dispersion equation, and accounts for diffusion, interception, gravitational settling, hydrodynamic interactions, and van der Waals forces.

In the RT model, η is calculated as

$$\eta = 4.04A_s^{1/3}\,\mathrm{Pe}^{-2/3} + A_s L_o^{1/8}\,R_{15/8} + 0.00338A_s S^{1.2}R^{-0.4} \quad (4)$$

where Pe is the Peclet number (Pe $= Ud_c/D$) and U is the approach velocity. A_s is a porosity-dependent term of the Happel model, defined as:

$$A_s = \frac{2(1-\gamma^5)}{(2-3\gamma+3\gamma^5-2\gamma^6)} \quad (5)$$

where $\gamma = (1-\theta)^{1/3}$. L_o is a dimensionless number that accounts for London-van der Waals attractive forces, and is defined as

$$L_o = \frac{4A}{9\pi\mu d_p^2 U} \quad (6)$$

where A is the Hamaker constant ($A = 1 \times 10^{-20}$ J), R is the ratio of the bacterial cell diameter to the sand diameter ($R = d_p/d_c$), μ is the viscosity of water, S is the dimensionless ratio of the bacteria's Stokes settling velocity to the stream fluid approach velocity ($S = u_{p,s}/U$); the settling velocity is given by

$$\mu_{p,s} = g\,\Delta\rho\,d_p^2/(18\mu) \quad (7)$$

and where g is the gravitational constant.

In the TE model, the collector efficiency is calculated as

$$\eta = 2.4A_s^{1/3}R^{-0.081}\mathrm{Pe}^{-0.715}N_{\mathrm{vdW}}^{0.052} + 0.55A_sR^{1.675}N_A^{0.125} + 0.22R^{-0.24}\mathrm{N}_G^{1.11}N_{\mathrm{vdW}}^{0.053} \quad (8)$$

where $N_G = u_{p,s}$,

$$N_A = \frac{2a_p^2\Delta\rho g}{9\mu U} \quad (9)$$

and

$$N_{\mathrm{vdW}} = \frac{A}{k_B T}, \quad (10)$$

where $\Delta\rho$ is the difference in densities of the fluid and particle phases, and all other parameters are the same as defined for the RT model. A key difference between the two models is that in the TE model, van der Waals forces (described by the van der Waals number N_{vdw} and the attraction number, N_A, the latter which represents the combined influence of van der Waals attraction and fluid velocity in particle collisions due to interception) are part of all of the particle deposition terms (diffusion, settling terms, and interception).

The calculation of the collision efficiency using the analysis described above is valid when a steady-state breakthrough concentration of bacterial cells is achieved. Unpublished research from our laboratory using the same bacterial strain and experimental protocol has confirmed that steady-state breakthrough is reached at this flowrate, and with this initial cell concentration. In addition, the bacterial breakthrough curves do not show evidence of blocking or ripening.

Nanoscopic studies

Preparing bacterial samples for AFM work. Glass slides were cleaned thoroughly with acids and hydrogen peroxide, and rinsed with generous amounts of ultrapure water, as described above. KT2442 cells were attached to clean glass slides, as described elsewhere (Camesano and Logan, 2000). Prior research has shown that biological activities are not disrupted by this attachment protocol (Lee *et al*., 1996; Wissink *et al*., 2000). After the bonding reaction, slides were transferred to a Petri dish containing water.

Treatments considered for AFM studies. Force measurements were performed on KT2442 cells that were centrifuged once, centrifuged multiple times (with no cellulase), and centrifuged multiple times during the process of enzymatic treatment with cellulase. Centrifugation conditions and the cellulase treatment protocol were the same as described for the microscopic (batch and column) studies.

Force analysis using AFM. Interaction forces were measured in ultrapure water at 25°C using silicon nitride cantilevers and an AFM (Digital Instruments Dimension 3100 with Nanoscope III controller). Silicon nitride tips were purchased from Digital Instruments (DNPS tips), and we measured the spring constant as 0.13 ± 0.02 N/m, using the Cleveland method (Cleveland *et al*., 1993). Tips were cleaned to remove organic contamination by exposure to ozone generated by ultraviolet light irradiation in an oxygen atmosphere for 1 min, just prior to use for each experiment (Tomitori and Arai, 1999).

A bacterium was imaged in tapping (intermittent contact) mode and brought to the center of the image frame before each force analysis. With the tip positioned over the center of a bacterium, the tapping motion of the tip was stopped and force cycles were captured. At least four measurements were performed on a single area of a bacterium and such measurements were performed on at least five bacteria for a given condition. Therefore, for each

condition, at least 20 force measurements were obtained. The origin of each force curve was determined by locating the region of constant compliance, based on the method proposed by Ducker *et al.* (1991). Very rarely, the data did not reveal a constant compliance region, indicating the tip was not making contact with the bacterial surface. In those cases, the data were discarded.

Although the force cycle contains information on both the "approach" and "retraction" of the AFM tip with the bacterial surface, we were mainly interested in retraction force cycles, since these provide quantitative information on the adhesive forces between the tip and the bacterial surface macromolecules. When the AFM tip has made contact with the bacterial surface biopolymers, often adhesion occurs between the two. The adhesive forces are observed until the tip is further retracted, and the polymer is released from the tip. The magnitude of the adhesion force at this pull-off time is termed the "pull-off force," and the distance between the sample and tip when pull-off occurs is the "pull-off distance." Each time an adhesion peak was observed, the adhesive force and the pull-off distance of the biopolymer were noted. Although 20–25 force cycles were examined for each condition, >100 adhesion events were observed for each condition. This is due to both multiple polymers from the bacterium contacting the tip and breaking off at separate locations, and single polymers present as loops or coils, making contact with the tip in more than one location. The data can be quantified as the discrete pull-off forces vs. pull-off distances. Since there will be spread in the data due to the natural heterogeneity of the sample and the dynamic nature of AFM force measurements, histograms are also used to describe the distribution of the force events.

We focused on the comparison of AFM adhesion data from retraction curves with bacterial retention data, rather than comparing the retention data with AFM approach curves. We previously explained that AFM retraction curves are more relevant for predicting microbial attachment affinities than AFM approach curves. Approach curves on numerous Gram-negative and Gram-positive bacteria and several fungal strains, with various probes and solutions, show strongly repulsive interactions (Camesano and Logan, 2000; Ahimou *et al.*, 2002; Van der AA *et al.*, 2002; Velegol and Logan, 2002; Abu-Lail and Camesano, 2003b; 2003c; Burks *et al.*, 2003; Vadillo-Rodriguez *et al.*, 2003; Emerson and Camesano, 2004). Yet despite the often-observed presence of strong steric repulsion, all of these microbes show varying attachment affinities with numerous substrates [see, e.g., Li and Logan, 2004; Pouliot *et al.*, 2005)]. While they are useful for providing information regarding polymer conformation, (Abu-Lail and Camesano, 2003b; Pouliot *et al.*, 2005), approach curves are less able to be used for the prediction of adhesion affinities. Since the present study concerns the comparison of adhesion affinities for different treatments of *P. putida* KT2442, we chose to present only the retraction curve analyses.

RESULTS

Microscopic retention and transport assays

Batch-type assays for quantifying bacterial retention, in which a substrate is immersed in the bacterial solution for a fixed period of time, removed, rinsed, and the cells either attached or in the suspended phase are counted, have been widely used to quantify bacterial retention (Jucker *et al.*, 1997; Smets *et al.*, 1999), especially for biomedical applications (Triandafillu *et al.*, 2003), due to their simplicity. When batch tests were conducted on *P. putida* KT2442 cells treated with cellulase, the resulting collision efficiency was decreased by $>40\%$ to 0.69 ± 0.13 (Fig. 2; where 0.69 refers to the mean of three measurements and 0.13 is the standard error of the mean), compared to either of the control cases. Both of the controls, centrifuged and filtered cells, experienced high collision efficiencies in the batch tests, and these values were nearly equal (1.19 ± 0.25 and 1.20 ± 0.23 for centrifuged and filtered cells, respectively). When diffusion is the transport mechanism for the bacteria, both groups of control cells behaved the same, and retention was less for the enzyme-modified cells. We also note that collision efficiencies greater than one have sometimes been observed, and may reflect a limitation in the ability to predict the number of collisions accurately (Logan, 1999). For the batch tests, a relatively high initial cell concentration was used, which could also have led to some cell-to-cell interactions that were not accounted for in the diffusion model.

For the control cells in the column assays, the α values for filtered and centrifuged cells were not significantly different from one another, according to a Mann-Whitney Rank Sum test (average values of 0.34 ± 0.06 and 0.45 ± 0.07, for filtered and centrifuged cells, respectively). Under comparable conditions with respect to cell preparation and treatment, collision efficiencies from batch tests were always higher than those measured under flow conditions in packed column assays (Fig. 2).

In the column tests, cellulase treatment again decreased the collision efficiencies compared with either of the control groups (Fig. 2). In the context of calculating the collision efficiencies (α values) for the column assays, we explored two models for calculating the collector efficiencies (η values), namely those proposed by Rajagopalan and Tien (1976) and Tufenkji and Elimelech

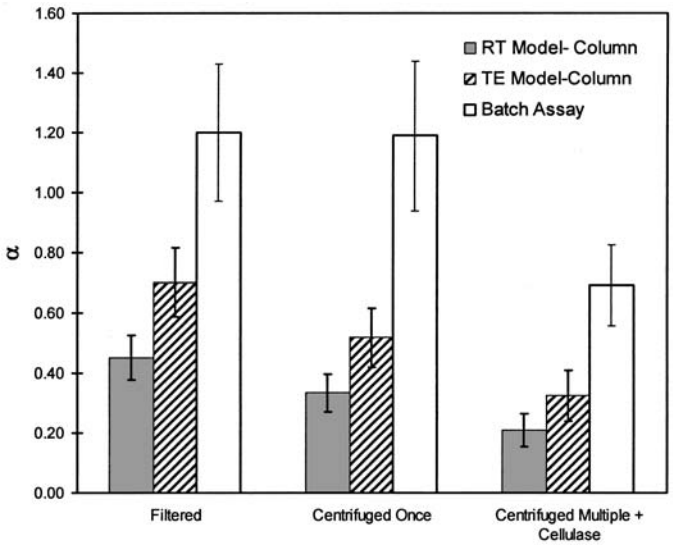

Figure 2. Comparison of collision efficiencies for *P. putida* KT2442 in batch and column assays, for different cell treatment protocols. Each bar shows the average collision efficiency, with the error bars representing the standard error of the mean. For the column assays, two models were used to calculate the collector efficiency, those proposed by Rajagopalan and Tien (1976) and Tufenkji and Elimelech (2004). Filtered cells and centrifuged cells each represent control cases. The centrifuged cells that were also treated with cellulase represent the "treated" condition, shown in the last set of bars on the right. For the batch and column assays, the collision efficiencies of filtered and centrifuged cells were not statistically different from one another (fails the Mann-Whitney Rank Sum test). The cellulase-treated cells were different from both of the controls, in batch and column assays (passes the Mann-Whitney Rank Sum test).

(2004). Both model approaches make use of the Yao *et al.* (1971) model to describe colloid filtration, but the RT and TE models differ in how the number of collisions between bacteria and soil particles are calculated. The TE model accounts for hydrodynamic interactions and van der Waals interactions that are important for Brownian particles. Regardless of the model choice, a similar trend was observed in the collision efficiencies with respect to cellular preparation. However, the collision efficiency values based on the TE model were ~55% higher than those calculated with the RT model.

Nanoscopic interaction forces

Adhesion forces between bacterial surfaces and AFM tips were distributed broadly in all cases, which likely reflect heterogeneity in the distribution and properties of macromolecules on the cellular surfaces (Fig. 3). Whether cells were centrifuged once or multiple times, the average adhesion force varied little ($F_{avg} = 1.05 \pm 0.07$ nN and 0.94 ± 0.08 nN for single and multiple centrifugation steps, respectively; the first number is the mean of >100 measurements and the deviation provided is the standard error of the mean). Treatment with cellulase decreased the average adhesion force by about half ($F_{avg} = 0.51 \pm 0.03$ nN). Examination of the distribution of adhesion forces observed between the biopolymers and the AFM tip reveals that high adhesion forces (>3.2 nN) were observed only for cells that had not been treated with the enzyme cellulase (Fig. 3). Cellulase treatment removed this population of highly adhesive polymers from the bacterial cells.

The average pull-off length of the polymers also changed depending on whether cellulase treatment was performed (data not shown). For cells not treated with the enzyme, but centrifuged either once or multiple times, the average pull-off distances were 272 ± 17.3 and 287 ± 13.1 nm, respectively (where the first number represents the mean of >100 measurements and the second number represents the standard error of the mean). For cells that underwent multiple centrifugation steps in conjunction with cellulase-treatment, the average pull-off distance decreased to 196 ± 9.0 nm, which is a reduction of ~30%. Caution must be exercised in interpretation of this changing pull-off distance. It may reflect both changes in the lengths of the polymers and changes in their conformations, and it is difficult to separate these two effects.

Biochemical analysis of macromolecules

We analyzed the suspending materials from the control bacterial solutions to determine the amount of glu-

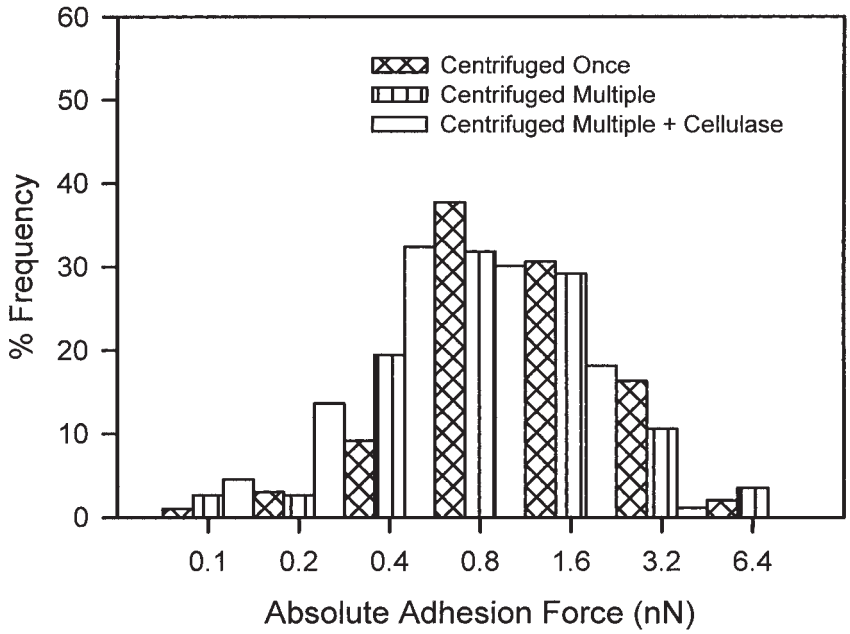

Figure 3. Adhesion forces measured between KT2442 biopolymers and the silicon nitride AFM tip, depending on cell preparation protocol. At least four measurements were performed on a single area of a bacterium and such measurements were performed on at least five bacteria for a given condition. Therefore, for each condition, at least 20 force measurements were obtained. Although 20–25 force cycles were examined for each condition, >100 adhesion events were observed for each condition. Histogram of the distribution of adhesion forces between KT2442 biopolymers and the AFM tips, depending on cell preparation protocol.

cose present. A similar test was conducted on materials that had been treated with cellulase. The anthrone assay can detect (by color changes) the presence of glucose monomers. The material treated with the enzyme contained much more glucose than the untreated materials from either control group. Based on triplicate measurements, cellulase treated materials contained $1.97 \times 10^{-2} \pm 1.19 \times 10^{-3}$ μg/L of glucose, compared to filtered materials ($1.59 \times 10^{-3} \pm 7.15 \times 10^{-4}$ μg/L) and centrifuged control materials ($2.18 \times 10^{-3} \pm 9.77 \times 10^{-4}$ μg/L). Cellulase-treated material contained approximately an order of magnitude more glucose than the nonenzyme-treated material. We cannot be certain that the material removed was entirely cellulose, since studies have shown that cellulase can act against related polysaccharides, including xylan (Kim *et al*., 1997), xyloglucans (Fry, 1995), and β-glucan (Bronnenmeier *et al*., 1995). In general, the family of cellulase enzymes, which includes 13 structural families, is active against other cellulose-derived molecules that contain fluorescent or chromophoric leaving groups (Barr and Holewinski, 2002). Although we cannot say whether all of the material degraded by cellulase was cellulose, we can clearly see that polysaccharide material was degraded to glucose monomers by the enzyme treatment.

DISCUSSION

Role of cellulose and similar polysaccharides on adhesion/retention of P. putida *KT2442*

For *P. putida* KT2442, cellulose or related macromolecules play an important role in cellular retention to glass and sand, and in the adhesion affinities for silicon nitride. Enzyme treatment clearly reduced the retention of the bacterium in batch and column assays, and the nanoscopic forces measured with the AFM were also decreased compared to untreated cells.

The chemical nature of cellulose may be unique in the way it mediates adhesion for *P. putida*. As far as we are aware, there are no reports of cellulose production by *P. putida* in the literature for comparison, although *Pseudomonas* species isolated from activated sewage sludge have been found to produce cellulose (Deinema and Zevenhuizen, 1971). In addition, several other bacteria and fungi, particularly those that naturally are present in

the rhizosphere, do produce cellulose, such as *Acetobacter xylinum* (Saxena *et al*., 1990) and *Agrobacterium tumefaciens* (Matthysse *et al*., 1995). The intrinsically adhesive nature of cellulose in bacteria may result from its biological role. For example, in *A. tumefaciens*, the cellulose fibrils help the bacterium attach strongly to plants, such as carrot cells (Matthysse *et al*., 1981). Extracellularly produced cellulose from *Pseudomonas* appears to help the flocculation of particles in wastewater (Deinema and Zevenhuizen, 1971; Cannon and Anderson, 1991).

The retention of *P. putida* to glass and sand, and the adhesion forces for silicon nitride, appear to be aided by cellulose or cellulose-like molecules. Cellulose is a neutral molecule and is not by itself particularly hydrophobic, but when more than one strand is present, cellulose does take on a hydrophobic nature. If multiple strands are present, the molecules align with one another to form extensive intramolecular and intermolecular hydrogen bonds that lead to the insolubility of cellulose in aqueous solutions and render the complex of molecules hydrophobic. This hydrophobicity may be important in influencing how the bacterium adheres to surfaces in water, and it can explain why *P. putida* cells with cellulose and related molecules present adhered to a greater extent than those in which these molecules had been removed. Although it may be possible to detect hydrophobic interactions with AFM experiments, we could not detect any long-range attractive forces from AFM approach curves on this strain (Abu-Lail and Camesano, 2003b). We attribute this absence to steric repulsive interactions masking any attractive forces in approach curves.

Role of cell separation protocols on biopolymer properties and adhesion/retention

We did not see an effect of gentle centrifugation on cellular retention or adhesive forces for *P. putida* KT2442, but this is probably due to the very low centrifugal force that was used ($<200 \times g$). Previous studies have noted that centrifuged bacteria behave differently than noncentrifuged cells. For example, Smets *et al.* (1999) examined *P. fluorescens* 2-79RL cultures that were centrifuged for 10 min at $10{,}000 \times g$. Centrifuged cells were retained less to glass slides in batch assays or to glass beads in packed column assays, compared to the noncentrifuged control cells. In a study designed to characterize the way in which cell preparation protocols affected cell surface analysis techniques, Pembrey *et al*. used $5000 \times g$ as the reference case, since it was known not to disrupt cell surfaces or alter values of cell surface parameters, such as electrophoretic mobility, hydrophobicity (quantified in the microbial adhesion to hydrocarbons test), or batch retention to a variety of substrates. In their study, treatment by centrifugation at $15{,}000 \times g$ affected many physicochemical properties and cell surface parameters for *Escherichia coli* and *Staphylococcus epidermis*.

Filtration, as we used it here, did not greatly affect cellular behavior. The collision efficiencies in the batch retention assay were essentially identical to those of the centrifuged cells. In the column assays, the collision efficiencies at first appeared to be different between the filtered and centrifuged cells (0.34 ± 0.06 and 0.45 ± 0.07, respectively), but statistical analysis demonstrated that the values from the triplicate experiments were not statistically different from one another (Mann-Whitney Rank Sum test). Therefore, it appears that either gentle centrifugation or backfiltering bacterial cells can be used as control cases for future experimental work in which cell separation from growth media is required.

Bacterial retention in batch and column assays

Batch retention experiments on bacteria have some benefits (rapid, many replicates can be performed, virtually any bacterial–substrate pair can be examined inexpensively), but can also lead to questions regarding the behavior of the bacteria upon passage through an air/liquid interface. As pointed out by Rijnaarts *et al.* (1993), if the hydrodynamic conditions of the experiments are not well controlled, batch retention assays can lead to results that are erratic or difficult to reproduce (Van Loosdrecht *et al.*, 1987a, 1987b). Despite potential problems, in one investigation of 12 bacterial strains, including *P. putida* mt2 and three other *Pseudomonas* strains, the level of detachment from glass in batch retention assays after passage of the substrate through an air/liquid interface was less than the standard deviation observed for replicate batch experiments (10–20%) (Rijnaarts *et al*., 1993). They found the disruption in the number of attached cells to be dependent on the substrate, since bacteria adsorbed to Teflon detached in much greater numbers upon exposure to the air/liquid interface, compared to those adsorbed onto glass. An investigation of *Staphylococcus epidermidis* and *P. aeruginosa* interacting with glass and polymer substrates demonstrated that if the substrates were rinsed with saline followed by ethanol before exposure to the air, no bacterial cells detached (Pitt *et al*., 1993). Yet when they skipped the ethanol rinse, bacteria detached from the substrates and reattached in clumps. In another investigation on *P. aeruginosa* and four other bacterial strains, the passage of an air bubble over a flow chamber did affect detachment of the cells, but it depended on the bacterial–substratum combination and the velocity of the air bubble, with important factors being the bacterial cell shape and the substrate hydrophobicity

(Gómez-Suárez *et al.*, 2001). These authors cautioned that batch studies involving rinsing and dipping of a substrate into a bacterial solution should be termed "bacterial retention" rather than "bacterial adhesion." In the present study, we did not find bacteria to be in clumps on the slides. Rather, they were well distributed on the glass even though the substrates had passed through an air/liquid interface. While we cannot assume that the velocity at which the slide passed through the air/liquid interface was the same in all cases, we observed a reasonable standard deviation on the number of attached cells/cm^2 for the triplicate batch experiments. Acknowledging potential problems associated with the interpretation of batch tests, we are careful to refer to the studies as "retention" assays, and not "adhesion" assays.

Upon comparison of bacterial retention behavior between the batch and column assays, we found that retention of the cells was much greater in the batch assays, with α values two to three times higher in the batch compared to the column studies. This is in contrast to prior research, which has shown that bacterial transport is much greater under convective (column) conditions, compared to the quiescent conditions of a batch experiment (Medilanski *et al.*, 2003; Rijnaarts *et al*., 1993). A possible explanation for the discrepancy is that the bacteria have a greater affinity for glass than for the sand grains, so hydrodynamics alone cannot account for the differences in retention behavior. Both the batch and column assays did demonstrate the same trends in the collision efficiencies with respect to cellular preparation, namely that similar values were observed for the two control cases (centrifuged and filtered cells), and substantially lower values were observed for the enzyme-treated cells.

Comparing bacterial retention with adhesion forces

Qualitatively, we saw similar trends in the correlation of the retention behavior of the bacteria between batch and column assays with direct force measurements at the nanoscale. An examination of data from many bacterial and some fungal species suggests there is a strong positive correlation between the collision efficiency in packed-column experiments and the force of adhesion measured with an AFM tip (Camesano *et al*., 2005). With only three data points from the present study, we cannot construct a quantitative relationship between the collision efficiencies and the adhesion forces based solely on this work.

However, some authors have recently attempted to calculate collision efficiencies directly from forces measured with the AFM (Cail and Hochella, 2003) or from energy calculations based on extended-DLVO theory (Dong *et al*., 2002b). Dong *et al*. (2002b) calculated collision efficiencies based on a theoretical analysis developed by Spielman and Friedlander (1974), in which the deposition of Brownian particles interacting with collectors is modeled in terms of attractive London-van der Waals forces and electrical double layer repulsion. For the extended-DLVO energy profiles they calculated for *Comomonas* sp. DA001, this technique predicted collision efficiencies on the order of 10^{-181}, which were much too low to be consistent with experiments. When we attempted to use this technique to predict collision efficiencies from our AFM force data, we calculated collision efficiencies of essentially zero, due to the highly repulsive interactions observed between *P. putida* KT2442 and the model surface. Although we did not show AFM approach curves in this study, in several of our previous studies on *P. putida* KT2442, we presented approach curves with highly repulsive interactions (Camesano and Logan, 2000; Abu-Lail and Camesano, 2003c). The lack of suitability of the Spielman and Friedlander model in describing our AFM force profiles reemphasizes that for *P. putida* KT2442 interacting with silicon nitride or similar surfaces, DLVO forces are not dominant compared to steric interactions and other specific types of interactions that arise due to surface polymers. An opportunity exists for future research to try and construct better ways of predicting bacterial retention or attachment on the basis of AFM direct force measurements.

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